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# Mechanisms of p53-mediated Intrinsic and Extrinsic Tumor Suppression

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# MECHANISMS OF P53-MEDIATED INTRINSIC AND EXTRINSIC TUMOR SUPPRESSION

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*To My Family!*

# ABSTRACT

p53 is a promising target for cancer therapy. However, the molecular basis of the p53 tumor suppression function remains incompletely understood. Thus, in this thesis, we focused on studies of the molecular mechanisms of p53-mediated tumor suppression.

Since p53 mainly functions as a transcription factor, we addressed whether it is the promoter binding pattern of p53 or its cooperation with different other transcription cofactors that determinates the transcription profile and the subsequent biological outcomes. We explored the genome-wide binding sites of p53 with ChIP-seq. By comparing the p53-bound sites in chromatin in breast cancer cells upon p53 activating compounds inducing different outcomes (nutlin: cell cycle arrest; RITA: apoptosis; 5-FU: cell cycle arrest), we found that the major binding patterns of p53 are similar, regardless of the stimuli and biological outcomes. We identified 280 novel p53 target genes by parallel analysis of gene expression. Further investigation revealed that the repression of several genes, including oncogene *AURKA*, by p53 could be enhanced by STAT3 inhibition. We also found that Sp1 is a co-regulator of p53 transcriptional response and apoptosis upon RITA. Our results emphasized the importance of cofactors in p53-mediated transcriptional response.

In paper II, we performed genome-wide shRNA screen to identify genes essential for p53-mediated apoptosis. Integration of these data with gene expression analysis lead to the identification of Sp1 as a key cofactor indispensable for the initiation of p53-mediated pro-apoptotic transcriptional repression, required for the robust apoptosis. However, Sp1 had no effect on neither induction of pro-apoptotic genes nor p53-mediated cell cycle arrest. Using ChIP-seq data in combination with ChIP-PCR for p53 and Sp1, we uncovered that p53-mediated pro-apoptotic transcriptional repression required the co-binding of Sp1 to p53 target genes. Further study revealed that MDM2-medated degradation of Sp1 serves to counteract p53-mediated transcriptional repression and apoptosis. This study helps to promote our understanding of the mechanisms of p53-mediated apoptosis and provides new targets and strategy for p53/MDM2-based therapies.

Recent studies suggest that p53 plays a role in modulating the anti-tumor immune response. In paper III we focused on studies of the mechanisms by which p53 regulates immune surveillance. Our results show that reactivation of p53 by the small molecule RITA stimulated NK cell-mediated killing of primary human tumor cells derived from metastatic cancers of different origins via p53-dependent induction of ULPB2, a ligand of NK cell receptor NKG2D. We further identified *ULBP2* as a direct transcriptional target gene of p53 with a p53 response element within its first intron, with which p53 regulates its transcription. Interestingly, we found that, without p53 activation, this promoter region was methylated. The de-methylation of this region is required for *ULPB2* induction by p53. Our studies provide a molecular evidence for the direct transcriptional control of immune surveillance upon pharmacological restoration of p53 function. This contributes to better understanding of the interaction between tumors and immune system, and opens up a possibility for novel approaches for p53-based anti-tumor immune therapy.

# LIST OF PUBLICATIONS

I. Nikulenkov F, Spinnler C*, Li H*, Tonelli C, Shi Y, Turunen M, Kivioja T, Ignatiev I, Kel A, Taipale J, Selivanova G.

**Insights into p53 transcriptional function via genome-wide chromatin occupancy and gene expression analysis.**

Cell Death Differ. 2012 Dec;19(12):1992-2002.

II. Hai Li, Yu Zhang, Anda Ströse, Yao Shi, Donato Tedesco, Katerina Gurova, Galina Selivanova.

**Integrated high throughput analysis identifies Sp1 as a crucial determinant of p53-mediated apoptosis.**

Manuscript

III. Li H, Lakshmikanth T, Garofalo C, Enge M, Spinnler C, Anichini A, Szekely L, Kärre K, Carbone E, Selivanova G.

**Pharmacological activation of p53 triggers anticancer innate immune response through induction of ULBP2.**

Cell Cycle. 2011 Oct 1;10(19):3346-58.

* equal contribution

# RELATED PUBLICATIONS NOT INCLUDED IN THIS THESIS:

IV. Spinnler C*, Hedström E*, Li H, de Lange J, Nikulenkov F, Teunisse AF, Verlaan-de Vries M, Grinkevich V, Jochemsen AG, Selivanova G.

**Abrogation of Wip1 expression by RITA-activated p53 potentiates apoptosis induction via activation of ATM and inhibition of HdmX.**

Cell Death Differ. 2011 Nov;18(11):1736-45.

V. Li H, Lakshmikanth T, Carbone E, Selivanova G.

**A novel facet of tumor suppression by p53: Induction of tumor immunogenicity.**

Oncoimmunology. 2012 Jul 1;1(4):541-543.

VI. Zawacka-Pankau J, Grinkevich VV, Hunten S, Nikulenkov F, Gluch A, Li H, Enge M, Kel A, Selivanova G.

**Inhibition of glycolytic enzymes mediated by pharmacologically activated p53: targeting Warburg effect to fight cancer.**

J Biol Chem. 2011 Dec 2;286(48):41600-15.

VII. Shi Y, Nikulenkov F, Joanna Zawacka-Pankau, Hai Li, Razif Gabdoulline, Jianqiang Xu, Sofi Eriksson, Elisabeth Hedström, Natalia Issaeva, Alexander Kel, Elias Arnér, Selivanova G.

**ROS-dependent activation of JNK converts p53 into an efficient inhibitor of oncogenes leading to robust apoptosis.**

Cell Death Differ. 2014 21(4):612-23.

* equal contribution

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| | |
|---|---|
| ActD | actinomycin D |
| ADCC | antibody-dependent cellular cytotoxicity |
| CDDP | Cis-diamminedichloroplatinum |
| CTD | C-terminal domain |
| CTLs | cytotoxic T lymphocytes |
| DBD | DNA binding domain |
| EMT | epithelial-mesenchymal transition |
| GOF | gain-of-function |
| KI | knock-in |
| LOF | loss-of-function |
| NCI | National Cancer Institute |
| NES | nuclear export signal |
| NK | nature killer |
| NLS | nuclear localization signal |
| NTD | N-terminal domain |
| PXXP | proline-rich domain |
| RE | response elements |
| RNAi | RNA interference |
| RNAPI, II, III | RNA polymerase I, II, III |
| shRNA | short hairpin RNA |
| TAD | transactivation domain |
| TSS | transcription starting sites |

# 1 INTRODUCTION

## 1.1 CANCER

Cancer is one of the top leading causes of human death. 7.6 million people died from cancer in 2008 worldwide according to the data taken from GLOBOCAN database (http://globocan.iarc.fr/). And this number is predicted to keep rising and will reach 13.1 million by 2030. The rising incidence of cancer is probably due to the extended lifespan, changes of lifestyle and environment and -partially- because of early diagnostics due to the advancement of cancer screen tests and diagnosis.

The oncogenic transformation can occur in nearly all types of cells from over 60 of the 78 organs in human body (http://www.cancerresearchuk.org/; http://www.organsofthebody.com/). Thus there are more than 200 different kinds of cancers identified in humans (http://www.cancerresearchuk.org/). Despite the great variety of cancers, there are several steps common for the development of all types of cancers.

The normal developmental process and the maintenance of multicellular organisms are dependent on the strict control of cell cycle and cell death. To initiate proliferation, firstly, the normal cells must receive the stimuli of extrinsic pro-growth factors, such as EGF or FGF, which bind to their receptors expressed on the surface of these cells. The activation of growth factor receptors in turn triggers the intracellular growth signaling via their tyrosine kinase domains to regulate the cell cycle and proliferation. However, cancer cells are commonly characterized by uncontrolled cell growth and the ability to invade from local mass to other parts of the body through the lymphatic system or bloodstream. Thereby, to initiate oncogenic proliferation, the cells must acquire the ability to proliferate independently of the stimuli of growth factors. Cancer cells mainly acquire this ability by 1) accumulating mutations involved in the activation of the downstream component of the pathways governing proliferation, which stimulates proliferation directly, 2) elevating the level of growth factor receptors expressed on cell surface, 3) inducing the self-activation of these receptors, or even by 4) producing growth factor itself, which will initiate the autocrine activation of growth factor and signaling thereby promote proliferation.

Besides, there are also several negative mechanisms inhibiting abnormal cell proliferation, such as growth inhibitor signaling mediated by pRB, cells contact inhibition via merlin, C/EBP alpha, p27, p21 and cell death programs by p53. So, to proliferate independently, cancer cells must also acquire mutations which can compromise these proliferation inhibitory, as well as pro-apoptotic mechanisms.

However, gaining the ability of self-sufficient proliferation is only the first step towards a true cancer cell. Normal cells do not express telomerase and can only divide for limited times then undergo senescence. Therefore, pre-oncogenic cells need to maintain their telomerase activity to become immortal.

In normal cells, energy is supplied via oxidative phosphorylation in mitochondria. Due to the uncontrolled proliferation, cancer cells need more energy than normal cells. Therefore, in cancer cells, besides retaining the oxidative phosphorylation at the similar level to normal cells, the activation of oncoproteins and malfunction of

tumor suppressors also initiate glycolysis which generates energy faster than oxidative phosphorylation, known as 'Warburg effect'. This process also provides enough substrate to the biosynthetic pathways which is curtail for cancer cell proliferation and division. However, despite of the high speed, the efficiency of ATP generation through glycolysis (2 ATP per glucose) is much lower than it via oxidative phosphorylation (36 ATP per glucose). The activation of oncoproteins and malfunction of tumor suppressors can also promote the formation of new blood vessel into tumor mass, known as 'angiogenesis', which supplies local tumor mass enough glucose and oxygen. Besides, the new vessels infiltrated into tumor mass also provide channels for cancer cells to migrate to distant parts of body and form secondary tumors.

In addition to the cell-intrinsic barriers mentioned above, an important cell-extrinsic barrier to oncogenesis is anti-tumor immune response. This immune barrier is essential for the initiation of oncogenesis. The infiltration of immune cells can be seen in nearly all tumors, which some times helps to create a favorable environment for oncogenesis in tumor development. However the majority of cancer cells can be eliminated efficiently by immune system. Only the cells containing mutations providing resistance to immune system can finally develop into tumor. In these tumors, the inflammation environment promotes oncogenesis by supplying factors enhancing survival, angiogenesis, metastasis and inhibiting cell death signaling (Hanahan and Weinberg, 2011).

To develop into cancer, cells have to obtain all characteristics described above, which takes a long time (Fig. 1). Impaired genome stability and capacity of DNA repair can contribute to the accumulation of these genetic abnormalities, leading to malignant transformation. These features are commonly seen in human cancer cells (Hanahan and Weinberg, 2011).

Taken together, features mentioned above are characterized as the hallmarks of cancer (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

Although the molecular basis of cancer are these genetic alterations that alter the cellular activities mentioned above, only approximately 5 to 10% of all cancers can be traced directly to inherited genetic defects (http://www.cancer.org/). The majority of cancers are caused by the accumulation of genetic alterations occurring throughout one’s lifetime. These genetic alternations can be caused by diverse factors from the environment, such as tobacco smoke, dietary factors, certain infections, exposure to radiation, exposure to certain chemicals, lack of physical activity, obesity and environmental pollutants (Anand, 2008). Thus, many cancers could be prevented by changing lifestyle, such as no tobacco smoking, having healthy diet, management of obesity, regular physical exercise, reducing exposure to radiation and chemicals, and vaccination against infection. However, the underlying mechanisms are complex and only partially understood.

Today, cancer is still mainly treated with radiation therapy or surgery followed by chemotherapy, which in many cases remain inefficient in prevention of cancer recurrence. Surgery is unable to eradicate invasive cancer cells and both radiation therapy and chemotherapy sometimes can initiate secondary cancer. Therefore, 90% of cancer deaths are caused by the metastasis, but not by a primary tumor. Although many complementary and alternative cancer treatments have been developed, the efficiency is

still limited. There is an urgent need to develop novel more specific and non-genotoxic therapies.

Studies of the response of National Cancer Institute (NCI) 60 human cancer cell lines to treatment by 86 clinically used anticancer agents treatment found that the therapeutic effect of these agents is strongly correlated with the p53 status and apoptosis, indicating the defect of p53-mediated apoptosis as a common and key mechanism of the resistance of cancer cells to drugs (Weinstein et al., 1997; Amundson et al., 2000).

Hence, the knowledge of the molecular basis of p53-mediated apoptosis in cancer cells is important for elucidating the nature of cancer and designing novel therapies.

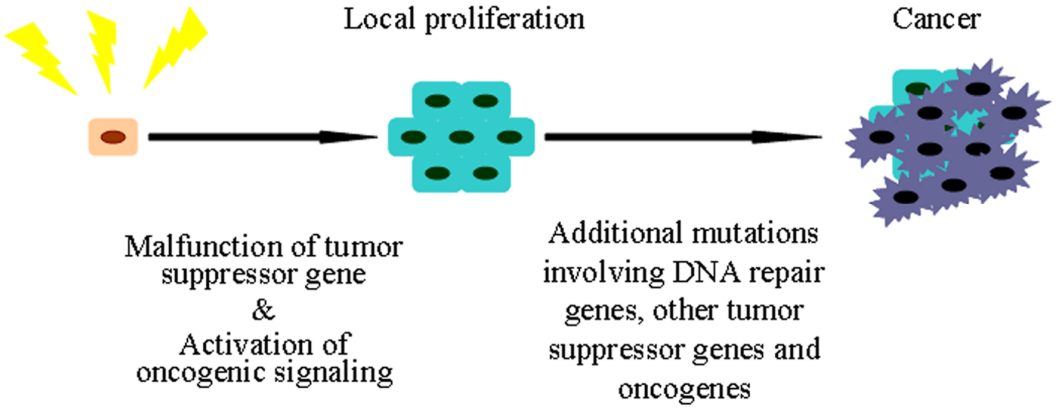


Figure 1. Oncogenesis is a process of the accumulation of genetic mutations of genes essential for cell death and proliferation. The malfunction of tumor suppressor gene and the activation of oncogenic signaling are required for the initiation of the development of cancer and leads to uncontrollable cell proliferation. Additional defects in DNA repair genes then allow cells to accumulate more mutations. Over time accumulated mutations can transform the primary tumor into a highly malignant, metastatic tumor.

## 1.2 P53

### 1.2.1 Discovery of p53: from an oncogene to a tumor suppressor

p53 (also known as tumor protein 53, TP53) is a protein encoded by the *TP53* gene in human and mouse, whose molecular weight is 43.7-kilodalton (kDa) based on calculation of its 393 amino acid residues, but it is 53 kDa according to its migration in SDS-PAGE. p53 is one of the most important tumor suppressors, since the inactivation of the p53 network is required for the development of nearly all human cancers (Vogelstein et.al., 2010).

p53 was discovered in 1979 as a cellular partner of the viral oncogenic protein large T-antigen during the studies of SV40 infection by different research groups (Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Melero et al., 1979; Smith et al., 1979). In the same year, DeLeo et al. observed high level of p53 protein in many types of mouse tumor cells, suggesting a connection between p53 and oncogenesis. Therefore, many studies were carried out to explore the effect of p53 on oncogenesis in the next few years and the results strongly suggested that p53 is an oncogene, since overexpression of p53 in normal cells can lead to oncogenic transformation (Eliyahu et al., 1984; Jenkins et al., 1984; Parada et al., 1984; Eliyahu et al., 1985). These findings greatly encouraged many investigators to focus their efforts on p53. However, the oncogenic role of p53 was soon questioned by a "two-hit" test performed in colorectal tumors, which surprisingly found that, in the majority of tumor cells, p53 is mutated (mainly point mutation) (Baker et al., 1989). Similar result was found using "two-hit" test in several other type of tumors (Nigro et al., 1989). This made it clear that p53, overexpression of which transformed normal cell into cancer cell as mentioned above, was mutated.

Up to now, p53 has been demonstrated to be the most frequently mutated gene in human tumors, and more than 25,000 mutations have been reported (http://p53.iarc.fr/). The observation of germline mutations of p53 in Li-Fraumeni syndrome patients, who are prone to the development of many type of tumors, provided a direct link between the tumor development and p53 mutation (Malkin et al., 1990; Srivastava et al., 1990). A genome-wide study of cancer-associated SNPs identified a SNP located in the 3' untranslated region of *TP53*, which results in impaired *TP53* transcript, as the one mostly associated with cancer among 16 million SNPs analyzed in 7790 prostate cancer cases, 1395 glioma cases and 4095 colorectal adenoma cases collected world wide (Stacey et al., 2011). The newest pan-cancer analysis of mutations associated with oncogenesis in 12 types of human cancers found that *TP53* is the most frequently mutated gene (42%) among the 127 genes significantly mutated in human cancers, followed by *PIK3CA* (17.8%) and *PTEN* (9.7%), and the mutation of *TP53* gene is significantly associated with patient survival (Kandoth et al., 2013).

What is the exact role of p53 in oncogenesis? After revealing the actual sequence of the wild type p53 gene, the biological outcome of wild type p53 overexpression was assessed in different cell lines, which established that the wild type p53, in contrast to mutant p53, repressed the growth of cancer cells *in vitro* (Eliyahu et al., 1989; Finlay et al., 1989; Yonish-Rouach et al., 1991; Shaw et al., 1992). The tumor suppression function of p53 was soon confirmed by an *in vivo* study with p53 knock-out (KO) mice, showing that mice deficient for p53 are more susceptible to oncogenesis (Donehower et al., 1992; Lowe et al., 1993). In contrast, mice with three copies of wild type p53 are more resistant to oncogenesis than normal mice (Garcia-Cao et al., 2002). These findings finally established the role of p53 as a *bona fide* tumor suppressor.

### 1.2.2 Biological activities of p53

Earlier studies of p53 have already found that the biological outcomes of wild type p53 overexpression vary among different cell lines: in some cell lines the

expression of wild type p53 triggered cell cycle arrest; whereas in other cell lines the expression of wild type p53 resulted in cell death rather than arrest (Diller et al., 1990; Mercer et al., 1990; Yonish-Rouach et al., 1993).

Recently, it became increasingly clear that p53 is at the hub of multiple signaling pathways and can be activated and regulated in response to many kinds of stresses, e.g. DNA damage, infection, oncogene activation, hypoxia, nutrient deprivation, heat shock and others (Fig. 2). Thus, upon different stimuli, activated p53 can induce various biological outcomes. Apart from the well studied outcomes of p53 activation (DNA repair, senescence, apoptosis, and cell cycle arrest), p53 can also affect other cellular processes, such as autophagy, metabolism, angiogenesis, immune response, and others (Meek, 1999; Miyakoda et al., 2002; Lavin and Gueven, 2006; Hu et al., 2007; Mathias et al., 2013), which may also have a role in its tumor suppressive activities (Li et al., 2012; Valente et al., 2013).

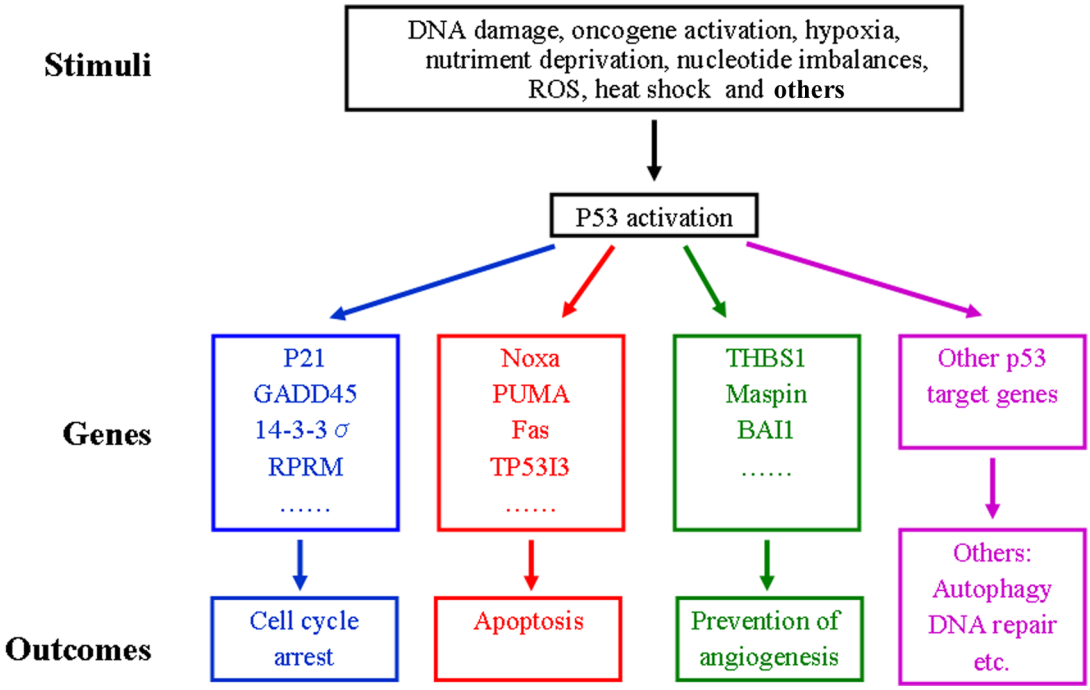


Figure 2. Diagram of the p53 tumor suppressor pathway. A wide range of stress stimuli activate p53 and subsequently lead to the expression of p53 target genes involved in diverse cellular functions.

The outcomes conferring the tumor suppressor function of p53 are involved in nearly every aspects of oncogenesis (Fig. 3):

**DNA repair:** DNA repair is essential for the prevention of oncogenesis, since it can prevent the mutations in genome. The importance of p53 and p53-dependent cell cycle arrest in DNA repair has been shown in both mouse cells (Smith et al., 2000) and human cells (Avkin et al., 2006). p53 can regulate DNA repair via its transcriptional control of DNA repair genes, such as *DDB2* (Hwang et.al., 1999), *XPC* (Adimoolam and Ford, 2002) and *Pierce1* (Sung et.al., 2010). Except above mechanisms, p53 can also directly control dNTP production via p53R2 (Tanaka et al., 2000).

**Cell cycle arrest:** in addition to halting the proliferation of cancer cells, cell cycle arrest is also required for p53-mediated DNA repair as mentioned above. p53 can induce cell cycle arrest through its control of the checkpoints of G1, G2 and M phases via its transcriptional control of genes such as *p21, ClnG, ClnD1, GADD45, 14-3-3σ* and *BTG2* (Amundson et al., 1998).

**Cell growth:** p53 can inhibit cell growth by regulating the levels of growth factors, growth factor receptors and key components of survival signalling. For example, p53 has been shown to inhibit the expression of FGF2, EGFR and PIK3CA, which are important for proliferation, resistance to apoptosis and angiogenesis (Galy et al., 2001; Singh et al., 2002; Astanehe et al., 2008; Bheda et al., 2008; Huang et al., 2011).

**Metabolism:** cancer cells shift its energy production type from mitochondrial respiration to aerobic glycolysis. p53 can repress many glycolytic enzymes and reduce the source of energy by inhibiting glucose uptake and lipid synthesis (Maddocks and Vousden, 2011; Berkers et al., 2013).

**Angiogenesis:** VEGF is an important growth factor for the initiation of angiogenesis. Study based on 833 breast carcinoma patients showed that the low p53 and high VEGF expression pattern correlates with bad outcome, indicating that p53 might have a important role in angiogenesis (Linderholm et al., 2000). Recent study found that p53 could either repress or promote VEGF expression in the presence or the absence of p21-Rb respectively (Farhang et al., 2013).

**Telomerase:** p53 can inactivate the activity of telomerase by direct transcriptional repression of hTERT in a p21-dependent manner (Shats et al., 2004).

**Inflammation and immune response:** some studies point out that p53 can suppress inflammatory response (Albina *et al.*, 1993; Messmer and Brüne, 1996; Rimessi et al., 2013) and promote anti-cancer immune responses by inducing chemotaxis of monocytes and cytotoxic T lymphocytes (CTLs) (Shiraishi *et al.*, 2000) and improving susceptibility of tumor cells to immune cells (Zhu *et al.*, 1999; Thiery et al., 2005). A recent *in vivo* mice model with switchable p53 expression shows that the tumor suppression by p53 in this model is innate immune system dependent (Xue et al., 2007; Lujambio et al., 2013).

**Metastasis:** besides the inhibitory effect on inflammation and angiogenesis, both of which can promote metastasis, p53 can also negatively regulate TGFβ-mediated epithelial-mesenchymal transition (EMT) (Termén et al., 2013).

**Epigenetic modification:** altered epigenetic modification is a common characteristic of cancer cells, which can reprogram the transcription profile of transformed cells. Recent studies suggest that p53 functions both upstream and downstream of epigenetic changes: e.g. on one hand, p53 can respond to the alternation of DNA methylation state, which promotes its transcriptional activity (Leonova et al., 2013); on another hand, wild type p53 can increase the acetylation of histones at p53 target genes, leading to enhanced transcription (Vrba et al., 2008), repress DNA 5'-cytosine-methyltransferases (DNMT) transcription, overexpression of which is correlated with many cancers, (Lin et al., 2010) and recruit HDACs to the promoters of p53 targets (Murphy et al., 1999).

**Cell death:** p53 has been shown to trigger death of cancer cells by induction of apoptosis, necrosis, senescence or autophagy (Amundson et al., 1998; Brown and Attardi, 2005; Chaabane et al., 2013; Qian and Chen, 2013; Kenzelmann et al., 2013). However, among these outcomes, senescence and autophagy can also prevent cell death and confer the resistance of cancer cells to anti-cancer treatments (Galluzzi and Kroemer, 2008; Altman and Rathmell, 2012; Jackson et al., 2012). Therefore, apoptosis is the most favorable outcome of p53 activation for cancer therapy.

The great variety of the tumor suppressor activities of p53 helps to explain the fact that *TP53* is the most frequently mutated gene in human cancer and also suggests the restoration of p53 tumor suppressor function in cancer therapies. However, the mechanisms of the cell fate decision upon p53 activation remain largely unknown.

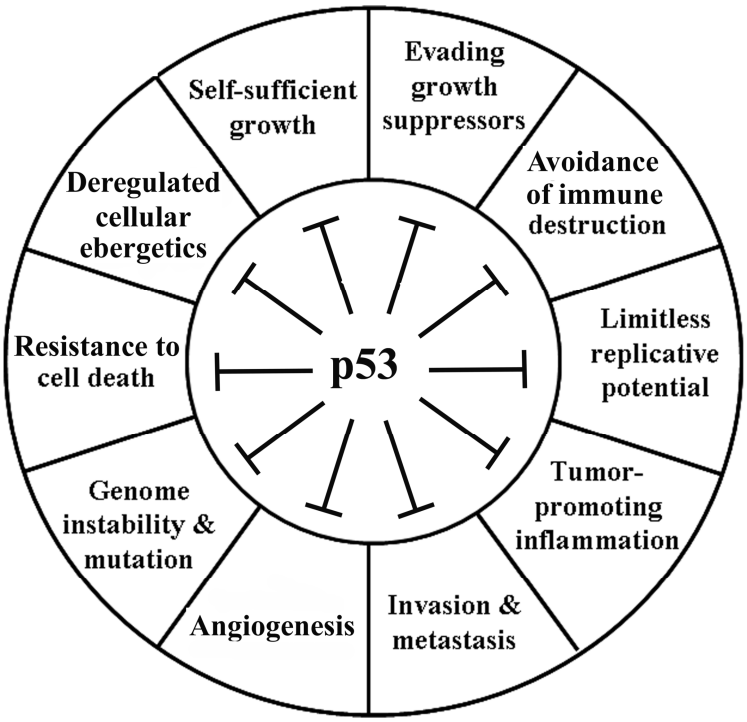


Figure 3. Tumor suppressor function of p53 targets the major hallmarks of cancer.

### 1.2.3 Malfunction of p53 in cancer

Malfunction of p53 pathway is required for the development of nearly all human cancers (Kastan, 2007). *TP53* mutations occur in almost every type of cancer and around 50% of human cancers harbor *TP53* mutations (Ahmed et al., 2010; Cyriac et al., 2013; Peller and Rotter, 2003). Mutant p53 can be detected at all stages of the process of oncogenesis, suggesting that the malfunction of wild type p53 may contribute to every phase of oncogenesis (Rivlin et al., 2011). 80% of *TP53* mutations are located within its DNA binding domain (DBD) (Olivier et al., 2002). 40.1% of them occur in six residues (R175, G245, R248, R249, R273, R282) (Cho et al., 1994; Levine, 1997). Mutations in DBD make p53 unable to bind to specific RE and regulate its target genes (Hollstein et al., 1991; Chao et al., 1994; Ko and Prives, 1996; Levine,

1997; Bullock and Fersht, 2001). Mutations affecting the C-terminal tetramerization domain can inhibit the formation of tetramers of p53, making it partially or completely deficient for specific DNA binding (Ko and Prives 1996). In addition to loss-of-function (LOF) effects of *TP53* mutations metioned above, many types of p53 mutations have been reported to gain additional oncogenic function (GOF) (Brosh and Rotter, 2009; Oren and Rotter, 2010). The concept of mutant p53 GOF was formally introduced in the study by Dittmer et al. in 1993, which showed that mutant p53 of both human and mouse origin could transform p53-null cells and increase the formation of colonies *in vitro* and tumorigenisis in mice. Today, various lines of evidence indicate that at least some p53 mutants affect the cancer cell transcriptome, increase genome instability (Gualberto et al., 1998; Caulin et al., 2007), suppress apoptosis via down-regulation of procaspase-3 (Lotem and Sachs, 1995; Wong et al. 2007), enhance cell migration by antagonizing p63 function (Adorno et al., 2009), promote tumor invasion through the inactivating Slug degradation (Adorno et al., 2009; Wang et al.. 2009) and enhance cell proliferation by interrupting wild type p53 function, like induction of *p21* (Duan et al., 2008).

In wild type p53 cancers, p53 pathway is also frequently abrogated. The most common mechanism is the deregulation of p53 negative regulators. Amplification of *MDM2* and *MDM4* (encoding MDMX) genes have been observed in many human cancers expressing wild type *TP53*. High levels of MDM2 or MDMX inhibit the tumor suppression function of wild type p53 or promots the degradation of the p53 protein (Perry, 2010). Malfunction of p53 in wild type p53 cells can be also achived by the deregulation of the positive regulators of p53, like homozygous deletions or promoter methylation of the gene encoding p14ARF, a protein that competes with MDM2 for binding to p53 (Midgley et al., 2000). Wild type p53 pathway can also be abolished by viral infection, via viral oncogenic proteins, such as E6 of human papilloma virus (HPV), EBNA1, EBNA3C and Gemin3 of EBV, E1B and E4ORF6 of adenovirus, Large T-antigen of SV40, which can promote p53 degradation, inhibit p14ARF, or block the DNA-binding affinity of p53 (Scheffner et al., 1990;Yew and Berk, 1992; Jiang et al., 1993; Scheffner et al., 1993; Chiocca, 2002; Cai et al., 2011; Frappier, 2012).

The possible mechanisms for the p53 pathway malfunction in cancer cells are summarized in Fig. 4.

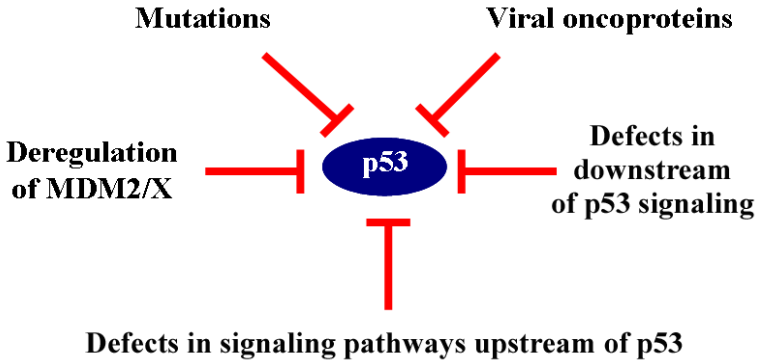


Figure 4. Mechanisms for the p53 pathway malfunction in human

### 1.2.4 Therapeutic targeting of p53 to combat cancer

Albeit inactive, the p53 protein is expressed in cancers, leading to the idea of p53 reactivation to combat cancer (Selivanova, 2010). However, although malfunction of the p53 pathway is a common feature of cancer development, the effect of p53 reactivation in established tumors remained unclear before 2006.

To address this question, Martins et al. assessed the effect of reinstatement of p53 on the maintenance of established lymphomas and survival of mice in a mouse model established by crossing switchable p53 knock-in (KI) mouse with a Emu-myc lymphoma model. Despite potential selection for p53-resistant tumors due to ARF inactivation or p53 mutation, reinstated p53 can be activated spontaneously by DNA damage signaling in established tumors and induce rapid apoptosis in p53-sensitive tumors and at least in some p53-resistant tumors. This study provides a strong evidence for the possibility of suppression of established tumors by endogenous and/or exogenous p53-activating pathways.

Similar study has been performed by Ventura A et al. in mouse model with controllable expression of endogenous p53 using a Cre-loxP-based strategy. These experiments also revealed the regression of established tumor by restoration of endogenous p53 in autochthonous lymphomas and sarcomas in mice. Importantly, they also claimed that there is no effect of p53 restoration on normal tissues (Ventura et al., 2007). Moreover, they observed that the biological outcome of restoration of endogenous p53 was tumor type-dependent, with p53-mediated apoptosis in lymphomas and p53-mediated senescence in sarcomas.

Another *in vivo* study addressing this question was performed in a liver carcinoma mice model with controllable endogenous p53 expression using RNA interference (RNAi). After the establishment of tumors, they shut down the p53 RNAi expression to restore p53 expression for a short period and observed complete tumor regression (Xue et al., 2007).

Profound *in vivo* suppression of different types of established tumors by reinstatement of p53, without affecting normal tissues, observed in above studies strongly promotes the idea of pharmacological restoration of p53 function to treat cancer.

According to National Cancer Institute database, there are more than 150 on-going clinical trials related to p53 (Cheok et al., 2011). p53 gene therapy in clinic has been approved in China (Lane et al., 2010). However, up to now, the reactivation of p53 in clinic is achieved by exogenous p53 overexpression or treatment with genotoxic compounds, which target both normal cells and cancer cells leading to side effects. Therefore, more and more investigators focused on the development of tumor specific p53-based therapies with minor side effects. Since half of human cancer cells harbor mutant p53 and the others carry non-functional wild type p53, p53 reactivation molecules can be divided into mutant p53-targeting therapies and wild type p53-targeting therapies (Fig. 5). Some of these molecules can target both wild type and mutant p53.

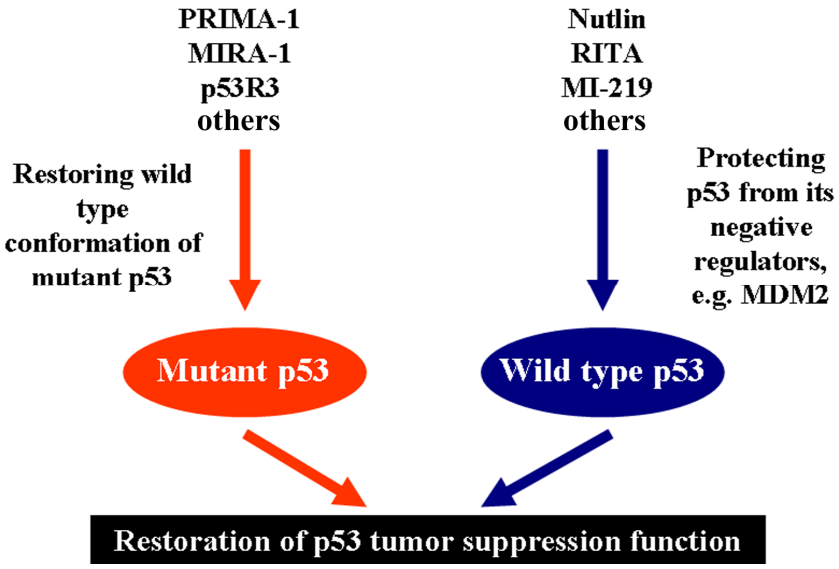


Figure 5. Therapeutic targeting of p53 to combat cancer

A number of molecules reactivating mutant p53 have been reported, such as CP-31398 (Foster et al., 1999), PRIMA-1 (Bykov et al., 2002), CDB3 (Friedler et al., 2002; Issaeva et al., 2003), MIRA-1 (Bykov et al., 2005), STIMA-1 (Zache et al., 2008), p53R3 (Weinmann et al., 2008), RETRA (Kravchenko et al., 2008), PhiKan083 (Boeckler et al., 2008) and SCH529074 (Demma et al., 2010). Among them, CP-31398 (Foster et al., 1999), PRIMA-1 (Bao et al., 2011), CDB3 (Luu et al., 2010), p53R3 (Weinmann et al., 2008), SCH529074 (Demma et al., 2010) have been reported to reactivate also wild type p53 via unknown mechanism. Several of these molecules have been shown to restore wild type p53 activity of mutant p53 via binding to DBD of mutant p53. However, the exact molecular mechanisms by which these molecules reactivate mutant or wild type p53 remain unclear.

Out of molecules mentioned above, only PRIMA-1 derivative Apr-246 is currently being tested in clinical trial (Selivanova, 2010). Phase I clinical trail has been done for APR-246 in patients with hematologic malignancies and prostate cancer to test the maximum-tolerated dose and safety (Lehmann et al., 2012). 60 mg/kg was set as maximum-tolerated dose, which did not induce serious adverse effects in patients. Results showed that Apr-246 successfully induced p53 signaling and lead to cell cycle arrest and apoptosis in several patients. Especially in one AML patient, the bone marrow blast was reduced from 46% to 26%. Some adverse effects, though completely reversible, were observed, including fatigue, dizziness, headache, and confusion.

A set of wild type p53-reactivating compounds have been developed targeting p53/MDM2 complexes to rescue p53 from the degradation by MDM2 via inhibiting the binding of MDM2 to p53, such as nutlin (Vassilev et al., 2004), MI-219 (Shangary et al., 2008), JnJ-26854164 (Johnson & Johnson, USA), PXn727 and PXn822 (Priaxon, Munich, Germany). Several compounds mentioned above are currently being tested in clinical trials. There are also molecules designed to target MDM4/p53 complex, such as SJ-172550 (Reed et al., 2010), stapled p53 helix peptide SAH-p53-8 (Bernal et al.,

2010) and recently reported stapled α-helical peptide ATSP-7041 which targets both MDM2 and MDM4 (Chang et al., 2013).

Nutlin is the first designed molecule targeting MDM2. Preclinical test for nutlin derivative RG7112 (RO5045337, Rroche) showed profound tumor repression effect in some type of cancer cells (Tovar et al., 2013). Importantly, RG7112 can be administrated orally. Phase I studies have been carried out in patients with hematologic malignancies and solid tumors for safety and efficacy test (http://clinicaltrials.gov/). A dose at 1500 mg/m$^2$/day was well tolerated by patients with soft tissue sarcoma and acute myeloid leukemia with some adverse events, such as nausea, diarrhea and vomiting. Administration of RG7112 activated p53 signaling in AML patients, as indicated by increased blood serum level of MIC-1, a product of the p53 target gene used as biomarker of p53 activation in serum, transcription of p53 targets including *MDM2*, *p21* and PUMA, and protein level of p53 itself in cancer cells. Apoptosis was induced by RG7112 in AML and CLL cancer cells in patients. Greater than 50% of reduction of blasts in peripheral blood or bone marrow was seen in around 40% of AML patients. Another extended phase I study with patients who took part in RG7112 clinical trails is currently going on to test the two years safety of RG7112 (http://clinicaltrials.gov/). In addition to tumor suppression function in hematological malignancies, RG7112 has also been shown to induce cell cycle arrest in normal cells, leading to the protection of normal tissue from the side effect of cytotoxic cancer therapeutic agents (Cheok et al., 2011). This effect of RG7112 provides a potential for 'cyclotherapy' in combination with chemotherapy, i.e., application of RG7112 in patients with p53 mutation (Lain, 2010).

Another strategy to block p53/MDM2 interaction is targeting wild type p53 directly. RITA (reactivation of p53 and induction of tumor cell apoptosis) is a small molecule found in our lab in a screen of the National Cancer Institute (NCI) library compounds. RITA can reactivate wild type p53 function by binding to p53, which disrupts the interaction between MDM2 and p53 and activates transcriptional transactivation of p53 both *in vitro* and *in vivo* without affecting normal cells (Issaeva et al., 2004). RITA has also been found to prevent the interaction between p53 and E6-associated protein E6AP and induce the accumulation of p53 and growth suppression in cells containing high risk HPV16 and HPV18 both *in vitro* and *in vivo*, since the binding of E6AP to p53 is required for HPV-E6-mediated degradation of p53 (Zhao et al., 2010). Binding of RITA to p53 can also prevent the interaction between p53 and Parc or iASPP, which inhibit p53 DNA binding (Issaeva et al., 2004). These data suggest that RITA induces a conformational shift in p53, which affects the binding of p53 to its partners. Besides, RITA can also restore wild type tumor supprresor function of mutant p53 (Zhao et al., 2010).

The alternative approach to rescue p53 function is to target regulators of p53 other than MDM2. Sirtuins negatively regulate p53 via de-acetylation (Haigis and Guarente, 2006). Tenovin 1 and its more soluble derivative Tenovin 6 are two sirtuin inhibitors identified by a p53-dependent reporter-based screen of a chemical library. Tenovins can inhibit Sirtuin 1, thus activating p53 by inducing acetylation of p53 at lysine 382 and suppressing the growth of various tumor cells both *in vitro* and *in vivo* without causing DNA damage (Lain et al., 2008).

Actinomycin D (ActD) can bind to the transcription initiation region of DNA and inhibit RNA polymerase-mediated transcription. It is originally used as an antibiotic drug in clinics and widely used as a transcription inhibitor in biology research. Due to its transcription blocking function, ActD has been used as a chemotherapy drug in the combination treatment of different type of cancers for a long time, especially in the treatment of Wilms tumor. While p53-independent tumor cell death induced by ActD has been reported (Merkel et al., 2012), recent studies have observed that, at low dose, ActD can stabilize p53 via ribosomal proteins-mediated inhibition of MDM2 activity in tumor cells and lead to tumor suppression in a p53-dependent manner, which is very similar with the action of nutlin (Zhang et al., 2003; Kalousek et al., 2007; Choong et al., 2009). Importantly, this low dose of ActD has synergetic effect in p53 activation with many well used chemotherapy drugs, such as Melphalan, Etoposide and Doxorubicin, which highlights the putative clinical usage of low dose of ActD in p53-based anti-cancer therapies (Choong et al., 2009).

The chemical structure of the p53-activating compounds used in the projects included in this thesis are shown in Fig. 6.

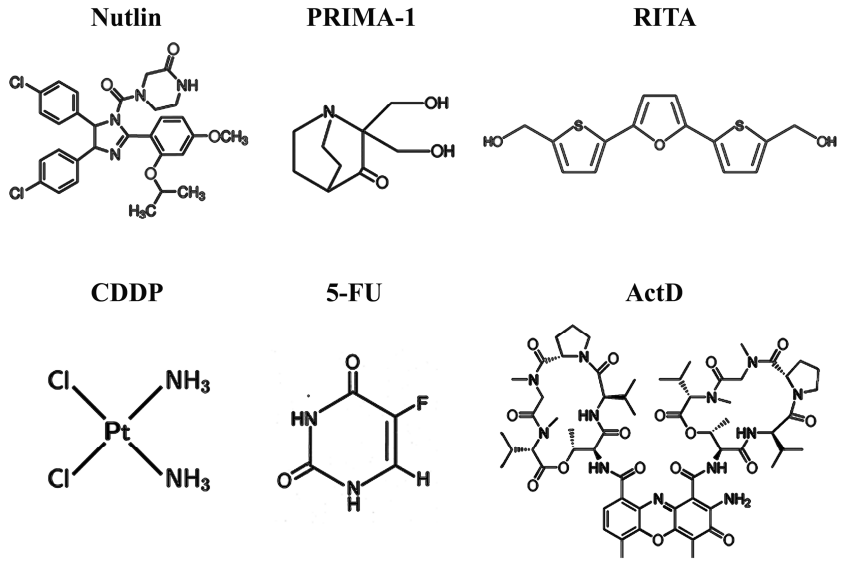


Figure 6. p53-activating compounds used in the projects included in this thesis: Nutlin, PRIMA-1, RITA, actinomycin D (ActD), cisplatin (CDDP) and 5-FU.

As mentioned before, p53 can respond to many disparate stresses and induce various biological outcomes. This makes the consequences of p53 reactivation difficult to predict. In line with it, the biological effects of many p53 reactivating compounds mentioned above have been shown to be cell type-dependent. For example, RG7112 has been shown to induce profound apoptosis in MHM, SJSA, LnCAP, 22Rv1 and A498 cells, but not in RKO, HCT116, A549, Lox and U2OS cells (Tovar et al., 2013). Lack of understanding of the molecular mechanism of p53-mediated biological outcomes can limit the success in p53-based therapies. More efforts to elucidate the molecular basis of how p53 regulates distinct biological outcomes are needed to develop p53-based therapies with controllable response.

### 1.2.5 p53 transcriptional activity

Wild type p53 is well known as a transcriptional activator (Farmer et al., 1992; Funk et al., 1992). The transcriptional activity of p53 is essential for its tumor suppression function (Jiang et al., 2011; Brady et al., 2011). p53 can interact with transcriptional machinery using its transactivation domain (Bargonetti et al., 1991; Kern et al., 1991). Its consensus binding site was revealed as RRRCWWGYYY (spacer n = 0-13) RRRCWWGYYY (R: adenine or guanine; W: purine; Y: pyrimidine). (Bargonetti et al., 1991; El-Deiry et al., 1992). Although the transcription-independent induction of apoptosis by p53 has been reported (Speidel, 2010), tumor suppression function of p53 is mainly due to its role as a transcription factor.

A number of genes has been identified as p53 target genes which are transcriptionally activated through the p53 response elements (RE) in their regulatory regions, including the cell cycle inhibitor *CDKN1A* gene encoding CDK inhibitor p21 (El-Deiry et al., 1993) and genes encoding the pro-apoptotic proteins *BBC3* (PUMA) (Nakano and Vousden, 2001; Yu et al., 2001) and, *PMAIP1* (NOXA) (Oda et al., 2000) and many others. Recent advancement of sequencing-based high throughput technologies allows the identification of hundreds of novel p53 target genes. Previous extrapolation of TF binding sites on chromosomes 21 and 22 using high density microarrays revealed estimated 1600 p53 binding sites in human genome (Cawley et al., 2004). Numerous studies found that the p53 RE could be located practically anywhere within the target gene, such as promoter (e.g., *CDKN1A*, *PMAIP1*), intron (e.g., *BBC3*, *PIG3* microsatellite RE), exon (e.g., miR-34a) and even enhancer far from the gene body (Riley et al. 2008). Besides binding to its REs with the central core DNA binding domain, p53 can also linearly diffuse on DNA/RNA with its C-terminal domain (CTD) (Palecek et al. 1997; McKinney et al. 2004; Liu and Kulesz-Martin 2006; Tafvizi et al. 2008), which enhances the recruitment of the TRRAP- containing histone acetyltransferase complex increasing p53 transcriptional activity (Barlev et al. 2001). Besides, p53 not only facilitates the transcription of RNA polymerase II (RNAPII)-transcribed genes, but also can repress RNAPI- and RNAPIII-mediated transcription (Cairns and White, 1998; Kim, 2011; Zhai and Comai, 2000).

It is more and more clear that there are hundreds of p53 target genes involved in many different cellular activities. Upon wide array of stresses, p53 can induce distinct transcriptional profiles and lead to different biological outcomes as described in last section. Many studies have been performed to explore the underlying mechanisms. These studies suggest that the transcriptional activity of p53 can be regulated by its level, sub-cellular location, post-translational modifications, DNA-binding ability and the cooperation with different transcriptional co-factors (Riley et al 2008).

*1) Regulation of the p53 protein level*

The protein level of p53 has been shown to determine the transcriptional activity of p53 and the subsequent biological outcome. It has been suggested that the high level of p53 induces apoptosis, whereas low level of p53 induces cell cycle arrest (Lai et al., 2007).

The protein level of p53 is mainly determined at the post-translational level via its proteolytic turnover through the interaction of p53 with MDM2 E3 ligase. In normal

non-stressed cells, the level of p53 is kept low due to the fast degradation mediated by MDM2. Upon different stress stimuli, p53 is protected from MDM2-mediated degradation via posttranslational modifications of both p53 and MDM2 or auto-poly-ubiquitination of MDM2 leading to the disruption of p53/MDM2 complex (Kruse and Gu, 2009; Vousden and Prives 2009; Marine and Lozano 2010). For example, the activation of some oncogenes can rescue p53 from MDM2-mediated degradation by inducing p14ARF, which can bind to MDM2 and inhibit the interaction between the MDM2 and p53, thereby activating p53 pathway (Kamijo et al., 1998). However, the increased level of p53 in turn activates MDM2 transcription, thus forming a negative feedback loop (Juven et al., 1993; Barak et al., 1994; Haupt et al., 1997; Phelps et al., 2003). The crucial role of MDM2 in p53 regulation was further confirmed by the rescue of embryonic lethality of MDM2 knockout mice in p53-null background (de Rozieres et al., 2000). Recently, MDM4, a close homologue of MDM2, has also been identified as a key determinant of the degradation of p53. In contrast with MDM2, MDM4 does not have E3 ligase activity, but enhances the degradation of p53 via binding to MDM2, which stabilizes it (Badciong and Haas, 2002; Kawai et al., 2007). Besides, some proteins, such as PIRH2, COP1 and CHIP, can also bind to p53 and mediate its degradation (Dornan et al., 2004; Esser et al., 2005; Leng et al., 2003). Among them, PIRH2 and COP1 are also p53 targets, which also form negative feedback loops. In addition, several proteins, such as RPS26 and p53 itself, have been shown to bind to the p53 mRNA 5′-UTR, which inhibited p53 mRNA translation (Takagi et al., 2005).

Since p53 mainly functions as a transcription factor, the cellular localization of p53 can also affect p53 transcriptional activity. The exposure of the nuclear export signal (NES) located at the C-terminal of p53 facilitates the export of p53 from the nucleus. In p53 tetramer NES is masked, which can prevent p53 export, while within the p53 dimers the NES unmasked, which allows p53 shuttling to the cytoplasm (Stommel et al., 1999). MDM2 can also contribute to shuttling of p53 from the nucleus to the cytoplasm by mediating the ubiquitination at the C-terminus region of p53 (Nie et al., 2007).

2) *p53 isoforms*

There are two promoters and 11 exons in human *TP53* gene. The combinations of alternative promoter usage and splicing can produce several p53 isoforms (Terrier et al., 2013). The full length p53 has a transactivation domain (TAD), a proline-rich domain (PXXP), a DNA binding domain (DBD), and a C-terminal domain (CTD), containing nuclear localization and export signals (NLS and NES), a regulatory domain and the tetramerization domain, as shown in Fig. 7. Due to a different combinations of promoter and exons, other p53 isoforms lack one or more parts of functional domains, leading to different activities. For example, due to the alternative splicing in intron 9, p53β has only a part of C-terminal domain, which leads to different binding affinities to p53 response elements (RE) located in some p53 target genes, such as *MDM2* and *CDKN1A* (Bourdon et al., 2005). Another example is Δp53, which is generated by the alternative splicing between exons 7 and 9. This isoform lacks part of the DBD and the NLS. Δ133p53 is initiated by the second promoter and lacks the TAD, PXXP, and part of the DBD. Both isoforms have impaired transcriptional activity (Terrier et al., 2013).

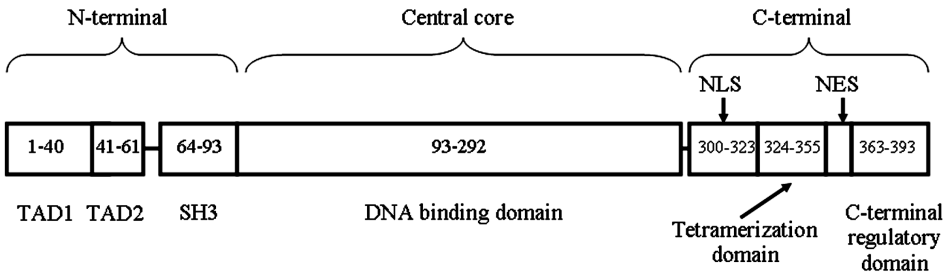


Figure 7. Functional domains of human p53. N-terminal portion consists of two transactivation domains (TAD1/2) and a Src homology 3-like (SH3) domain. The transactivation domain is required for transcriptional function and interacts with various transcription factors, acetyltransferases and the MDM2 ubiquitin ligase. The SH3 domain is a proline-rich domain required for interaction of p53 with SIN3, which protects p53 from degradation (Zilfou et al., 2001). The central core is made up primarily of the DNA-binding domain and the C-terminal end contains nuclear localization and export signals (NLS and NES), a regulatory domain and the tetramerization domain. Numbers indicate residues.

3) *Post-translational modifications*

In cells under stress, p53 protein can be activated by stress signals. The stress signals can mediate post-translational modifications of p53 via a number of protein kinases, such as protein kinases involved in the genome integrity checkpoints (CHK1/2, ATR, ATM, CAK, DNA-PK and TP53RK) and protein kinases of MAPK family (ERK1/2, JNK1 to 3, p38 MAPK) (Olsson et al., 2007). The post-translational modifications of p53 are essential for the modulation of p53 activity and subsequent determination of the biological outcomes of p53 activation (Bode and Dong, 2004; Dai and Gu, 2010; Brooks and Gu, 2011). For example, in response to DNA damage, p53 protein can be phosphorylated by ATM (Westphal, 1997; Banin et al., 1998; Canman et al., 1998) and Chk2 (Tominaga et al., 1999; Shieh et al., 2000), the master kinases of cell cycle checkpoints, which not only stabilize p53 protein by preventing the binding of MDM2 to p53 but also promote the recruitment of transcriptional cofactors to p53, like p300/CBP which can acetylate the C-terminal end of p53, allowing p53 to bind to DNA (Ionov et al., 2004; Vakhrusheva et al., 2008). Besides, Histone deacetylases (HDAC)-1, -2, and -3 and MTA2/PID and SirT2 can deacetylate p53 and subsequently inhibit p53 transcriptional activity (Juan et al., 2000; Luo et al., 2001). The diversification of the post-translational modifications of p53 also allows p53 to bind to different cofactors and to lead to various biological outcomes.

4) *Protein-protein interactions*

p53 can interact with more than 100 proteins. Many of these interactions have been shown to selectively alter p53's transcriptional program and modulate the binding affinity of p53 to its RE in target genes (Beckerman and Prives, 2010; Resnick-Silverman and Manfredi, 2006). p53 homologue p63 can bind to the REs similar to p53 in pro-apoptotic genes *BAX* and *Noxa* in E1A-immortalized mouse embryo fibroblasts.

This was suggested to facilitate the binding of p53 to these genes and subsequent induction of the transactivation of these genes and apoptosis by p53 (Flores et al., 2002). The binding of ASPP1 or ASPP2 to p53 can promote p53-mediated apoptosis by enhancing the p53 binding to the promoters of pro-apoptotic genes *BAX* and *PIG-3* (Samuels-Lev et al., 2001). In contrast, the binding of iASPP to p53 inhibits p53-mediated apoptosis (Bergamaschi et al., 2003; Bergamaschi et al., 2006). However, it is difficult to explain how p53 can bind DNA upon simultaneous binding to p63 and p73, as well as ASPP1 and ASPP2, since they bind to the p53 DBD.

Strap (stress-responsive activator of p300) is activated upon DNA damage by ATM and Chk2 kinases and is a key co-regulator of the p53 response, which can antagonize MDM2 and facilitate the recruitment of JMY and p300 to p53 targets (Shikama et al., 1999; Demonacos et al., 2001; Demonacos et al., 2004; Adams et al., 2008; Coutts et al., 2009). HIPK2 can mediate the phosphorylation of p53 at ser46 and promote the co-recruitment of HIPK2/p300/p53 complex to the promoters of apoptotic genes (Hofmann et al., 2002; Puca et al., 2009). Besides, a number of other proteins, such as transcription factors SMADs (Elston and Inman., 2012) and Sp1 (Gualberto and Baldwin, 1995; Bocangel et al., 2009; Lin RK et al., 2010), have been reported to interact with p53 at its targets, affecting the transcriptional activity of p53.

Sp1 is a ubiquitously expressed transcription factor which binds to GC-rich motifs in promoters and regulates the expression of a large number of genes involved in diverse cellular processes, including cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage, and chromatin remodeling (Tan and Khachigian, 2009; Chu, 2012). Sp1 can function as a pioneer factor and provide a platform for recruitment of protein complexes involved in transcription initiation and chromatin remodeling (Thomas et al., 2007; Davie et al., 2008; Huang and Xie, 2012), with an estimated more than 12,000 binding sites in human genome (Cawley et al., 2004). Interestingly, overexpression of Sp1 has been shown to induce apoptosis in different types of cell lines in a manner involving the transcriptional activity of Sp1 and p53 (Deniaud et al., 2006; Chuang et al., 2009). Therefore, in normal cells, the level of Sp1 should be under tight control as high level of Sp1 can trigger apoptosis in cooperation with p53. However, overexpression of Sp1 has been observed in many different types of human cancers (Zannetti et al., 2000; Wang et al., 2003; Sankpal et al., 2011), indicating the existence of unknown mechanism protecting cancer cells from Sp1-mediated apoptosis.

**Transcriptional repression by p53**

In addition to the initiation of the transcriptions of its targets, p53 can also repress the transcription of many genes, although the biological significance remains unclear. Several mechanisms of p53-mediated transcriptional repression have been reported.

(1) p53 binds directly to its RE and recruit co-repressors, like mSin3a, HDAC1, 2 and 3, to the target genes, such as *MAP4* and *STATHMIN* (Murphy et al., 1999), *HSP90-beta* (Zhang et al., 2004) and *C-MYC* (Ho et al., 2005).

(2) p53 can inhibit gene expression by inducing a repressor protein. One example is *CDKN1A*, a well-known direct transcriptional target of p53 (Lohr et al.,

2003). The protein encoded by *CDKN1A*, p21, can inactivate E2F-mediated transcription by inhibiting CDK-dependent phosphorylation of RB protein (Xiong et al., 1993; Niculescu et al., 1998; Delavaine and La-Thangue, 1999; Harbour and Dean, 2000).

(3) Another mechanism of p53-mediated transcriptional repression is competing for DNA binding with transcriptional activator. For example, in response to hypoxic stress, p53 binds to its RE at *AFP*, which displaces the binding of HNF3, the transcriptional activator of *AF*, leading to the repression of *AFP* expression (Lee et al., 1999). Similar mechanism is involved in the regulation of a number of other p53-repressed genes without p53 RE. p53 binds to these genes via protein-protein interactions. For example, *cyclin B2* promoter contains NF-Y binding site but does not have p53 binding site. But p53 can bind to the promoter of *cyclin B2* via the interaction with NF-Y. This protein complex can then recruit HDAC1, leading to the repression of *cyclin B2* (Imbriano et al., 2005).

**High throughput (HTP) technologies for addressing p53 biology**

The 33 years studies of p53 (Fig. 8) transformed our understanding of the p53 role from an oncogene to a tumor suppressor; revealed the nature of p53 as a transcription factor and the effects of transcriptional and posttranscriptional regulation of p53 on its activity; it has demonstrated the complexity of p53 network and the variety of biological processes in which p53 is involved. The numerous studies on p53 during these 33 years greatly improved our understanding of several principles underlying tumorigenesis, such as the difference between oncogenes and tumor suppressor genes; the molecular basis of tumorigenesis; and the molecular link between environmental stimuli and cell responses.

Despite of the huge number of publications on p53, we still have much to learn. The most important question to be answered perhaps is the mechanism of the cell fate decision by p53 activation. Answering this question will allow us to use p53-based therapies more efficiently and in a controlled manner.

p53 is well known as a transcription factor. However, because of the complexity of p53 network, it is not easy to fully understand the molecular mechanisms of cell fate decision by p53. On one hand, the gene transcription is regulated by multi-dimension mechanism in human cells: expression, epigenetic modifications, different combination of transcription factors, microRNA, long non-coding RNA, co-regulation by the interaction between DNA and DNA or RNA and DNA, and others. On another hand, human cells usually have alternative signaling to trigger the same cellular activity, therefore it is hard to identify the role of a single factor or signaling in p53-mediated cell fate decision by loss/gain-of-function-studies. Thus, to fully understand the molecular basis of p53-meidated outcomes, we need to study the multi-dimensional complex network as a whole. Recent development of the genome-wide HTP technologies on different molecular levels (i.e., epigenetics, proteomics and transcriptomics) may provide us such possibility.

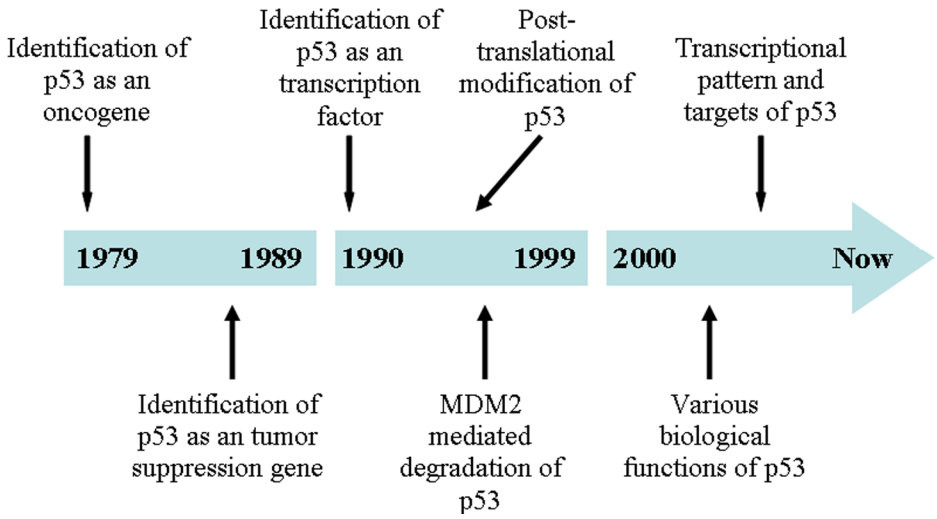


Figure 8. Timeline of p53 studies.

HTP technologies include:

MALDI-TOF MS to explore genome-wide gene expression profile on protein level

Whole genome sequencing (WGS) for mutations detection

Genome-wide loss-of-function screen using pooled siRNA or shRNA libraries

DNA methylation array for genome-wide DNA methylation detection

RNA-seq and cDNA microarray for exploring genome-wide transcriptional profile

ChIP-seq based methods are used to determine 1) genome-wide occupancy by specific transcription regulating factor or factor complex (ChIP-seq), 2) the interaction among different parts of chromatin, which are co-regulated in clusters, such as enhancer-promoter and promoter-promoter (end sequencing based ChIP-seq) or 3) the interaction between RNA and genome (like CLIP-seq, RIP-seq, ChIP-RP-seq and RICh-seq).

However, there are some challenges which need to be addressed.

Recent advanced MALDI-TOF MS can identify around 5000 different proteins in one run. However, because of the huge diversity of the levels of different proteins in cells, the signals from the low abundant proteins are usually too low to be used for difference comparison among groups. This limits the number of reliable signals used in advanced analysis.

All studies using sequencing-based high throughput technologies contain four basic steps: sample preparation, sequencing, primary data analysis and secondary data analysis. There are some common technical problems for these studies, which I summarize below.

1) The difference in sample materials, protocols and antibodies used during sample preparation and the difference in the machines and strategies used for sequencing can affect the final results.

2) Different platforms used for transforming the image files to sequences may generate sequence results with different qualities due to the different compression on image files.

These two issues can be addressed by unifying the procedure in the future.

3) Before doing any other analysis, all sequences generated must be aligned to a reference genome or other sequences. Great amount of sequences can be generated by the new generation sequencing method today. However, the quality and coverage of current reference sequences may not be sufficient to map all sequences correctly, leading to reduced quality and abundance of the information obtained.

4) Peak calling process is usually performed before doing functional analysis. However, different programs or even the same program with different criteria can lead to different results in the next functional analysis. One of the limitations for the development of statistical method is the limited number of repeats of sequencing, due to the high cost. More reliable analysis methods can be developed based on more repeats of sequencing for each sample with the development of faster and cheaper sequencing technology.

5) Most of reference sequences have not been fully annotated yet, especially like variant splicing and non-coding regions in genome, which limits the interpretation and lowers the significance of sequencing data. Therefore, more functional studies of these sequences are still needed.

6) Software for functional analysis used today differ in statistical methods and database used. Thereby, analysis performed with different software may give different predictions. Even analysis performed at different time with the same software may give distinct results due to the continuing update of databases employed by the software.

7) Because of the heterogeneity of tumor, it is difficult to isolate pure population of cancer cells. Therefore, the clinical and pre-clinical cancer samples usually have contamination with normal cells, which partially covers the molecular characteristics of cancer cells. This problem can be solved by using single cell sequencing. This developing new technique can sequence thousands of cells per week and up to 1000 cells per lane.

Thereby, all these issues mentioned above should be taken into account during data analysis today. The main conclusions of analysis should be always validated experimentally if it is possible.

## 1.3 IMMUNOSURVEILLANCE

The correlation between immune response and tumor suppression was first shown by Coley at the end of 19th century that tumor regression was frequently seen in cancer patients with bacterial infection or with injection of heat-inactivated bacteria or bacterial culture supernatants (Coley, 1991). But the tumor suppression function of the immune system was first predicted by Paul Ehrlich in 1909 based on the phenomenon

that the incidence of cancer in humans is quirt low and immune system is the main defense system of human beings (Ehrlich, 1909). When his paper was published, it was not taken seriously, since tumors develop in people with functional immune system and the immune system of cancer patients normally still functions well. Fifty years later, following the development of the clinical application of transplantation, this notion came back into people's view. To protect implanted tissue from the rejection by host immune system, patients must receive immunosuppression agents to inhibit their immune system temporarily. However, together with the success of transplantation, the initiation of secondary cancer was frequently seen in immunosuppressed patients. Since the donors are healthy and cancer free, the most possible reason of these cancer incidents after transplantation is the repression of immune system. Considering the difference between malignant cells and host normal cells, Burnet proposed that cancer cells should present some cancer-specific antigens which could be recognized by immune cells and mediate their elimination by immune system (Burnet, 1957).

The first tumor specific antigen which can be presented on the surface of tumor cells by HLA molecules and mediates CTL response in humans was identified in 1991 (van der Bruggen et al., 1991). Up to now, 403 tumor specific antigens which can initiate CTL response in humans have been identified (Vigneron et al., 2013). Dr. Thomas addressed this question in another way: given that cancer usually occurs at a later stage of human life, this disease must be under the control of the defense machinery, immune system. He further pointed out that the mechanism should be similar to that of homograft rejection (Thomas, 1959). Burnet and Thomas described the tumor suppression function of immune system they proposed as 'Immunosurveillance'.

In the following years, there were endless debates of the reality of this hypothesis. For example, on one hand, the infiltration of immune cells had been seen in nearly all tumors and cancer patients have T cells recognizing their own cancer cells; on another hand, although many immunoactivation stimuli had been tested in clinical trails, there was no firm evidence showing any of them giving patients clinical benefit. Thus without a direct evidence from animal model, this hypothesis could not be widely accepted.

In 1974 and 1975, using immunologically deficient mice (CBA/H strain, nude athymic), Stutman and Outzen et al. concluded that the immune system had no effect on oncogenesis, since that no difference in tumorigenesis was seen between nude mice and the wild type mice for either spontaneous nonviral tumors or tumors induced by methylcholanthrene (MCA) (Stutman, 1974; Outzen et al., 1975). This temporarily silenced the long debate for another 15 years. However, several studies at the beginning of this century not only found that the 'nude' mice Stutman used were only partially immunologically deficient since innate immune cells in those mice could still prevent oncogenesis, but also demonstrated the tumor suppression effect of immune system (Smyth et al., 2001b; Dunn et al., 2002).

But why tumor can rise in people with functional immune system? A classical experiment has been performed with RAG2-/- mice, which lack not only T and B but also NK-T cells (Shankaran et al., 2001). The authors induced sarcomas with MCA in both RAG2-/- mice and wild type (WT) hosts. When they transplanted cancer cells developed in RAG2-/- mice to RAG2-/- mice or WT hosts, they found oncogenesis in

all RAG2-/- mice but only 60% of WT hosts, suggesting the tumor suppression effect of immune system. However, when they transplanted cancer cells developed in WT mice to RAG2-/- mice or WT hosts, no tumor suppression was seen in any mice regardless of genetic background. This finding raised a hypothesis that, due to the tumor heterogeneity, there may exist some tumor cells that are resistant to immune responses, resulting in incomplete elimination of cancer cells by immune system, and these remaining cells can finally develop into tumor (Fig. 9). Therefore, in addition to tumor suppression function, host immune system also participates in selecting and editing cancer cells so that some cancer cells can escape from immunosurveillance (Dunn et al., 2004).

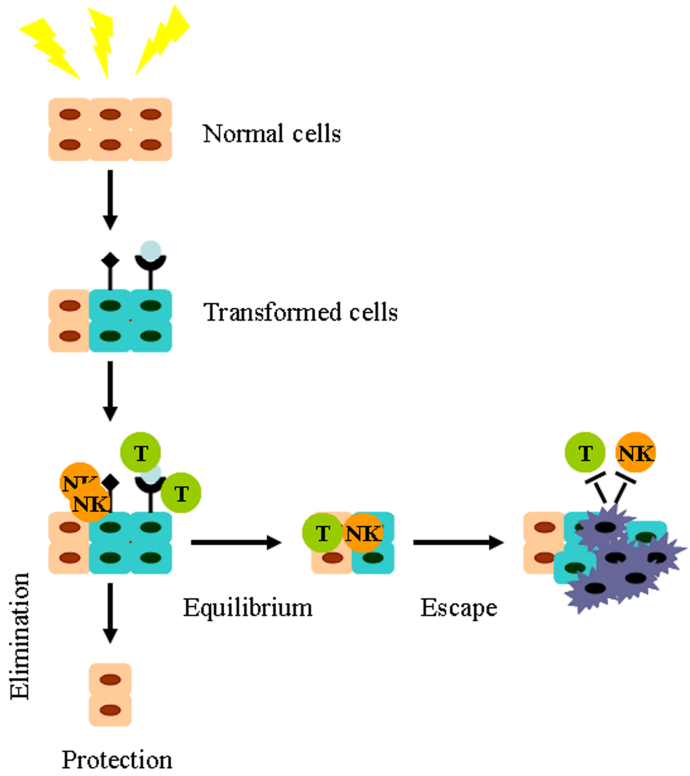


Figure 9. The three phases of the immunosurveillance. At early stages of oncogenesis, transformed cells may express distinct tumor-specific markers and initiate the anti-cancer immune response, which may eradicate the developing tumor and protect the host from tumor formation. However, if this process is not successful, the tumor cells may enter the equilibrium phase where they may be either maintained chronically or immunologically sculptured to produce new populations of tumor variants. These variants may eventually evade the immune system by a variety of mechanisms.

This dual role of immune system is named as 'Immunoediting'. More and more efforts have been done to understand how these cancer cells escape from the killing by immune system. Today we know that cancer cells can escape from immune system via at least three mechanisms:

1) the reduction of immunogenicity in tumor cells by reducing or totally losing molecules involved in antigen presentation, such as human leukocyte antigen (HLA) class I/II molecules, TAP, LMP and β2-microglobulin (Algarra et al., 2000; Marincola

et al., 2000; Seliger et al., 2000; Shankaran et al., 2001; Takeda et al., 2002), or by downregulating the expression of tumor specific antigen (Spiotto et al., 2004).

2) the immunosuppression by cancer cells via releasing immuno-suppression molecules such as TGF-β, IL-10, soluble NKG2D ligands (Kim et al., 2005; Groh et al., 2002) or VEGF which not only initiates angiogenesis but also inhibits the maturation of dendritic cells and promotes the infiltration of myeloid-derived suppressor cells (MDSCs), leading to suppression of anti-tumor immune responses (Alfaro et al., 2009).

3) the suppression of immunocompetence by cancer cells via elevating the expression of ligands which can target immuno-inhibitory receptors expressed on immune cells, for example HLA-G, a ligand highly expressed on cancer cell surface, which can inhibit NK cells, T cells, B cells, macrophages and dendritic cells through binding to its receptors expressed on these immune cells, such as KIR2DL4/p49, ILT-2 and ILT-4 (Urosevic and Dummer, 2008; Wilczyński and Duechler, 2010).

Therefore, the enhancement of immunosurveillance via targeting these three pathways will provide potential ways to combat the escape of cancer cells from immune control.

Anti-tumor immunity has been shown to be effective in inhibiting the growth of established tumors (Alyea, 2000; Velligas et al., 2002; Zhang et al., 2003). Recent studies showed that the repression of established tumors by anti-tumor immunity could be achieved by modulating host immune cells. Ipilimumab is an antibody specifically targeting CTLA4. The binding of Ipilimumab to CTLA4 blocks the inhibitory signal of cytotoxic T lymphocyte and leads to activation of T cell-mediated anti-tumor responses (Korman et al., 2006). Ipilimumab has been approved by the FDA for advanced melanoma therapy. Trastuzumab is the first antibody approved by FDA for cancer therapy. It can induce antibody-dependent cellular cytotoxicity (ADCC) in breast cancer. Co-stimulatory activation of natural killer (NK) cells with CD137 agonistic antibody could improve the repression of breast cancer cells by trastuzumab both *in vitro* and *in vivo* (Kohrt et al., 2012); the enhanced *in vivo* repression of established tumor has been also achieved via combination of CD137 agonistic antibody with oncolytic vaccinia virus (John et al., 2012). These studies demonstrate the clinical potential of combination of immune stimulation with tumor-targeted therapies to enhance the efficiency of cancer therapy.

In addition to direct activation of immune cells, many efforts have also been focused on the modulation of immunogenicity and immuno-regulating molecules. However, most of these studies are based on the application of antibodies, which is inefficient in many cases in clinic (Scott et al., 2012). Further studies of the mechanisms of immunosurveillance may help to develop novel therapeutic approaches to combat cancer.

### 1.3.1 p53 and Immunosurveillance

A principle issue of immunosurveillance needed to be addressed is the connection between 'cellular intrinsic tumor suppression mechanism' (tumor suppressors) and 'cellular extrinsic tumor suppression mechanism' (anti-tumor immune responses).

One of the most important tumor suppressors which protect us from cancer is p53. The malfunction of p53 signaling is required for the development of most cancers. p53 has been known to suppress oncogenesis by inducing cell cycle arrest, apoptosis, autophagy, senescence and necrosis (Menendez et al., 2009). Recent years' studies show that p53 is also involved in many other physiological activities, including immune responses (Vousden and Prives, 2009). For example, Super-p53 mice (with three copies of wild type *TP53* gene) are not only resistant to oncogenesis but also have stronger anti-viral immunity as compared with normal wild type *TP53* mice (García-Cao et al., 2002; Munoz-Fontela et al., 2005), providing an *in vivo* evidence for the effect of p53 on immune response. *In vitro* activation of p53 has been shown to suppress inflammatory response (Albina et al., 1993; Messmer and Brüne, 1996). Mice with p53-deficiency in intestinal epithelial cells show increased inflammatory response, associated with enhanced EMT and tumor invasion (Schwitalla et al., 2013). Strikingly, an *in vivo* study in mice with controllable p53 showed that tumor regression by p53 restoration in this model was pro-inflammatory cytokine production- and innate immunity-dependent, with infiltration of innate immune cells around tumor cells containing activated p53 (Xue et al., 2007). Their further investigation shows that, upon p53 restoration, cytokines produced by the tumor cells undergoing senescence induce differentiation of macrophages toward M1-state which can eliminate senescent cells; in contrast, cytokines produced by the p53-deficient tumor cells induce differentiation of macrophages toward M2-state which can promote the survival of tumor cells (Lujambio et al., 2013). These results suggest a cell autonomous function of p53 to suppress tumorigenesis through modulation of anti-tumor immune responses.

In addition to the anti-inflammation effect, p53 can also improve susceptibility of tumor cells to immune cells by promoting antigen presentation to immune cells (Zhu *et al.*, 1999; Thiery et al., 2005). Recently, Toll-like receptor 3 (TLR3), a crucial innate immune receptor, was identified as a target gene of p53, suggesting that p53 can affect immune response via direct regulation of gene transcription (Taura et al., 2008). p53 can also promote cell surface expression of major histocompatibility complex I via direct transcriptional control of endoplasmic reticulum aminopeptidase 1 (Wang et al., 2013), which provides another link between p53 function and the immunosurveillance.

Comparison of the gene transcriptional profile between human breast cancer patients with wild type or mutant p53 revealed that half of the genes differently expressed between patients with different p53 status were involved in immune responses, indicating a profound effect of p53 on immunity (Miller et al., 2005). However, none of these immune genes has been reported to be involved in p53-related signaling. Thereby, above findings we summarized are just a tip of the iceberg of the effects of p53 on anti-tumor immune responses. Further exploration of the mechanisms of p53-mediated modulation of immune responses may increase the probability to achieve a durable therapy by activating both 'cellular intrinsic tumor suppression function' and 'cellular extrinsic tumor suppression function' of p53.

### 1.3.2 NKG2D system

In humans, NKG2D ligands mainly include MICA, MICB, ULBP-1, -2, -3, and -4, which are expressed at low levels in normal cells, but highly expressed in cells under stresses, such as upon viral infection and oncogenesis. These ligands are

expressed on the surface of cancer cells and can be recognized by an immunoactivating receptor NKG2D, which is expressed on the surface of many immune cells, including NK cells, CD8+ T cells and γδ T cells. The binding of these NKG2D ligands to NKG2D receptor activates NKG2D receptor-positive cells and induce both innate- and adaptive- immune responses.

As cancer cells often repress the expression of MHC class I molecules to avoid recognition by immune cells, NKG2D ligands play fairly important roles in the activation of anti-tumor immune responses. The crucial role of NKG2D system in immunosurveillance against tumors has been confirmed by the higher sensitivity to both spontaneous and induced oncogenesis of *klrk1-/-* NKG2D-defecient mice, which otherwise have normal immune cell populations (Guerra et al., 2008). In mice, all NKG2D+CD8+ T cells infiltrate in tumor tissue, in contrast with other T cell populations, suggesting the specific tumor reorganization function of NKG2D+CD8+ T cells. This further confirmed the importance of NKG2D system in anti-tumor immunosurveillance (Gilfillan et al., 2002; Choi et al., 2007). Transplantation of cancer cell line into nude mice and wild type mice showed that, compared with cancer cells transplanted to nude mice, cancer cells transplanted to wild type mice expressed significantly lower level of NKG2D ligands (Gasser et al., 2005). The similar observation has been also seen in humans (Lakshmikanth et al., 2009). These studies strongly suggest a high efficiency of NKG2D system in tumor regression *in vivo*. Positive clinical correlation between NKG2D ligand expression and survival has been seen in patients with many different types of cancers (Conejo-Garcia et al., 2004; Poggi et al., 2004; McGilvray et al., 2009). Recent studies suggest the restoration of NKG2D ligand expression on tumor cell surface as a very promising approach for anti-tumor immunotherapy (Sutherland, et al., 2006; Raulet and Guerra, 2009).

In order to develop such approach, elucidation of the molecular mechanism regulating NKG2D system is needed. However, to date, there are only few studies elucidating the mechanisms regulating NKG2D ligand expression. A study in HCT116 colon cancer cell line showed that the expression of ULBP2 (one of NKG2D ligands) kept low due to the activation of RAS signaling and DNA methylation (Sers et al., 2009). They further revealed that blocking of RAS pathway by inhibition of MAPK or DNA de-methylation by knock-out DNMTs could dramatically increase ULBP2 expression and enhance ULBP2-mediated killing of HCT116 cells by NK cells *in vitro* (Sers et al., 2009). Besides, inhibition of histone deacetylases (HDACs) has also been shown to promote the expression of NKG2D ligands, sensitizing tumor cells to NK cell-mediated lysis (Diermayr et al., 2008; López-Soto et al., 2009). DNA damage signaling is commonly activated in cancer cells. It has been shown to induce the expression of NKG2D ligands on tumor cells (Gasser et al., 2005). It has been further demonstrated that this effect is ATM- and ATR-dependent and the expression of ATM and phosphorylation of Chk2 were necessary for the induction of NKG2D ligands (Gasser et al., 2005). However, oncogenic factors, such as c-Myc and Ras, which induced transformation of cells and initiated DNA damage signaling in their model, were not necessary for the induction of NKG2D ligands by DNA damage signaling. p53, which can function at both up- and down- stream of DNA damage signaling was also not required for the induction of NKG2D ligands in their model. However, a controversial result has been obtained in Eμ-Myc mice with lymphoma, demonstrating that the expression of NKG2D ligands on lymphoma cells was determined by the

balance between oncogenic factor, c-Myc, and tumor suppressors, p53 or Arf (Unni et.al., 2008). The authors suggested that the control of the expression of NKG2D ligands required the presence of both oncogenic factor and tumor suppressor in lymphoma cells, since the increased expression of NKG2D ligands they observed could be abolished by complete loss in either oncogenic factor or tumor suppressor (Unni et.al., 2008).

Interestingly, a recent study by Dr. Raulet DH.'s lab using mice with 'switchable' p53 showed that the elimination of senescent tumor cells by *in vivo* restoration of p53 was dependent on NKG2D system (Iannello et al., 2013). Although the upregulation of NKG2D ligands by DNA damage signaling has been suggested to be p53 independent (Gasser et al., 2005), the expression of NKG2D ligands may still be regulated by p53, since p53 activation can induce DNA damage signaling (Ahmed et al., 2011; de Lange et al., 2012). Thereby, it is possible that p53 activation induces NKG2D ligand expression indirectly via the induction of DNA damage signaling. Further investigation of the effect of p53 activation on the expression of NKG2D ligands and the underlying molecular mechanism may contribute to the development of more efficient therapeutic approach which can initiate both cell-autonomous tumor suppression and NKG2D system-mediated killing of cancer cells by p53 activation.

# 2 THESIS AIMS AND RESULTS

## 2.1 PAPER I

### *Insights into p53's transcriptional function via genome-wide chromatin occupancy and gene expression analysis*

Fedor Nikulenkov, Clemens Spinnler*, Hai Li*, Claudia Tonelli, Mikko Turunen, Teemu Kivioja, Alexander Kel, Jussi Taipale, Galina Selivanova

* equal contribution

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Key words: p53, ChIP-seq, repression, Sp1, STAT3, microarray

Aim: to obtain a genome-wide map of p53 occupancy upon activation by different compounds leading to distinct biological outcomes.

Pharmacological reactivation of the p53 tumor suppressor is a promising strategy for anti-cancer therapy due to the high potential of p53 to elicit apoptosis or growth arrest in cancer cells. However, the outcome of pharmacological activation of p53 is hard to predict, which can limit the clinical application of p53-targeted therapies. To elucidate the mechanisms underlying regulation of cell fate by p53, genome-wide p53 occupancy was performed using ChIP-seq method in breast cancer MCF-7 cells treated with three different p53-activating compounds: RITA, nutlin, and 5-FU. Treatment with these compounds lead to different biological outcomes: apoptosis, cell cycle arrest or a combination of both, respectively.

Surprisingly, analysis of p53-bound DNA sequences revealed that, regardless of stimuli and biological outcomes, most of p53 binds to the same sites in chromatin. These data suggest that p53 binding to promoters *per se* is not sufficient to regulate transcription and emphasizes the role of p53 cofactors.

Combined analysis of ChIP-seq data and gene expression data identified 280 novel p53 target genes, 214 of which are induced and 66 were repressed after nutlin treatment. p53 binding motif analysis suggested that only the minority of repressed genes contained p53 RE, while the majority of activated genes contained p53 consensus motif. These data suggest that repression occurs via different mechanism, i.e. either through indirect p53 binding or through motifs which are significantly different from the p53 consensus.

In addition to these possibilities, p53 might regulate the transcription of some genes far from its binding sites via 'looping', since we found that the location of many p53 binding sites was far from the TSS of its targets (more than 10 kb).

Besides, we also found that the frequency of p53 occupancy at the promoters of repressed genes was lower than that at the promoters of induced genes. This difference might due to the different mechanisms of transcription initiation and repression: transcription activation needs a constant binding of transcription activators, whereas

transcription repression only requires a transient presence of transcription repressors (Ptashne, 2011).

Among the 280 novel p53 targets we identified, we have chosen *AURKA* encoding Aurora kinase A as an example of a p53-repressed gene and *SEI1* encoding Sertad 1 as an example of a p53-induced gene for further validation. Aurora kinase A is an oncogene which expression correlates with poor outcome of anti-cancer therapies (Yang et al., 2011). Our studies suggest that STAT3 can be an important cofactor antagonizing p53-mediated repression, including *AURKA*. STAT3 can bind to the promoter of *AURKA* and antagonize p53 thus blocking p53-mediated repression.

We also identified another transcription factor, Sp1, as a co-modulator of p53, which confer p53-mediated transcriptional profile upon RITA treatment. Depletion of Sp1 can prevent RITA-induced growth suppression but has no effect on cell cycle arrest by nutlin.

This study emphasized the importance of co-operating and antagonizing transcription factors in p53-mediated transcriptional response.

However, the factors required by p53 to induce specific cell fate and the underlying mechanisms by which these factors determine cell fate together with p53 remain unknown. Since p53 can bind to more than 100 different proteins, genome-wide loss-function-study should be employed to address these issues.

## 2.2 PAPER II

***Integrated high throughput analysis identifies Sp1 as a crucial determinant of p53-mediated apoptosis***

Hai Li, Yu Zhang, Anda Ströse, Yao Shi, Donato Tedesco, Katerina Gurova, Galina Selivanova

Key words: p53, Sp1, transcriptional repression, apoptosis

Aim: to identify factors essential for p53-mediated apoptosis.

We performed an integrated analysis of genome-wide shRNA screen in combination with gene expression analysis and genome-wide p53 occupancy analysis to further elucidate the molecular mechanism of p53-mediated apoptosis.

We established an experimental system for studying cell type- and stimulus-specific responses to p53 activation in three wild type p53-expressing human cancer cell lines of different origin, MCF-7 breast carcinoma cells, SJSA osteosarcoma cells, HCT116 colorectal cancer cells, and an isogenic derivative of HCT116 cells with p53 deletion (HCT116 *TP53-/-* cells), upon the treatment with five well-studied p53-activating chemical compounds, nutlin, RITA, actinomycin D (ActD), Cis-diamminedichloroplatinum (cisplatin, CDDP) and 5-FU. Finally, MCF-7 cells and HCT116 cells treated with RITA were chosen to further investigate cofactors needed for p53-dependent apoptosis. Nutlin, which predominantly induced cell cycle arrest in MCF-7 and HCT116 cells was selected for comparison.

To identify factors crucial for p53-mediated apoptosis, we performed genome-wide short hairpin RNA (shRNA) screen in MCF-7 cells treated with RITA using a pool of lentiviral shRNAs constructs, composed of 27,290 shRNAs targeting 5,046 known human genes. The results indicate that p53 simultaneously regulates a number of alternative signaling pathways to mediate apoptosis. Thus, blocking of a single signaling pathway does not sufficiently prevent p53-mediated apoptosis. Pathway analysis of genome-wide shRNA screen identified totally eighteen pathways synthetic lethal with p53-mediated apoptosis. Half of these pathways were shown to be involved in p53-mediated apoptosis for the first time, which might provide new targets for future development of combination therapies.

Further analysis of functional protein association with resistance nodes, depletion of which rendered cells resistant to RITA-induced apoptosis, indicated Sp1 as a central modulator of p53-mediated apoptosis.

Functional studies via different methods further confirmed the key role of Sp1 in p53-induced apoptosis in a cell type- and stimuli-independent manner, while no effect of Sp1 on p53-mediated cell cycle arrest was detected.

Parallel analysis of genome-wide shRNA screen data and gene expression data identified Sp1 as a key cofactor indispensable for the initiation of p53-mediated pro-apoptotic transcriptional repression, required for the robust apoptosis. In contrast, Sp1 had no effect on the induction of pro-apoptotic genes.

Combined analysis of chromatin occupancy data and gene expression data uncovered that p53-mediated pro-apoptotic transcriptional repression required the co-binding of Sp1 to p53 target genes.

Further investigation revealed that MDM2-medated degradation of Sp1 serves to counteract p53-mediated transcriptional repression and apoptosis, suggesting that protection of Sp1 from the degradation by MDM2 is crucial for p53-mediated apoptosis, which provides a new target and strategy for p53 based therapies.

Taken together, our results emphasize the importance of Sp1 in p53-mediated apoptosis, which contributes to the elucidation of the mechanistic basis of p53-mediated apoptosis and provides new targets and strategy for p53-based therapies.

There are still some questions which remain unclear.

Why p53 reactivated by different pharmacological compounds have different abilities of binding to Sp1?

It has been shown that p53 can be phosphorylated at Ser46, a phosphorylation site important for p53-mediated apoptosis, by RITA treatment but not by nutlin treatment (Tovar et al., 2006; Ahmed et al., 2011; de Lange et al., 2012; Ma et al., 2012). It is possible that the different post-translational modifications of p53 lead to distinct binding abilities of p53 to Sp1 and subsequent different transcriptional profiles induced by p53.

What is the mechanism of the different regulation of MDM2 level by p53 reactivated by RITA or nutlin?

Given that the transcript of MDM2 was induced by both RITA and nutlin, RITA or nutlin may have different regulation of the post-transcriptional modification of MDM2. Interestingly, the pathway analysis of p53 target genes co-repressed by p53

and Sp1 after RITA treatment showed that the 'Top1' pathway was ubiquitin-mediated proteolysis, indicating that p53 and Sp1 might co-regulate MDM2 on post-transcriptional level.

Besides, we found that many chromatin modifying enzymes were under the co-regulation by Sp1 and p53, which could lead to profound changes of chromatin structure and gene expression profiles. Further investigation of the effect of p53 activation on chromatin modifications may help to better understand the mechanisms of p53-mediated apoptosis.

Based on our data, we suggest a model describing the impact of Sp1 for p53-mediated apoptosis (Fig. 10).

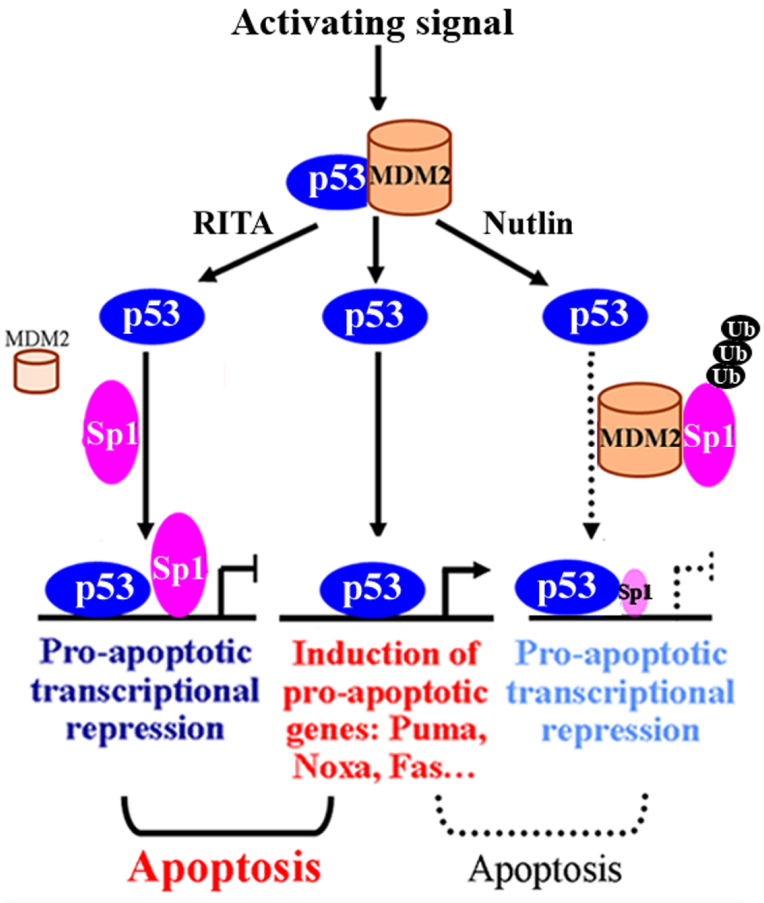


Figure 10. Upon stimuli, p53 becomes activated and induces pro-apoptotic genes, such as Puma, Noxa and Fas. In cells with low level of MDM2 activated p53 mediates pro-apoptotic transcriptional repression via recruiting Sp1 to its response elements in the p53 target genes, which together with the induction of pro-apoptotic genes trigger efficient apoptosis. However, in cells with high level of MDM2, Sp1 is degraded by MDM2. Without recruitment of Sp1, p53 fails to confer pro-apoptotic transcriptional repression and, although pro-apoptotic genes are induced, efficient apoptosis is not triggered.

## 2.3 PAPER III

***Pharmacological activation of p53 triggers anticancer innate immune response through induction of ULBP2***

Hai Li, Lakshmikanth Tadepally, Cinzia Garofalo, Martin Enge, Clemens Spinnler, Andrea Anichini, Laszlo Szekely, Klas Kärre, Ennio Carbone, Galina Selivanova

Cell Cycle. 2011 Oct 1;10(19):3346-58.

Key words: p53, pharmacological activation, DNA methylation, tumor suppressor, cancer immunotherapy

Aim: to address the mechanism by which pharmacologically activated p53 regulates NKG2D-mediated immune surveillance.

Cell-intrinsic barrier mediated by tumor suppressors and cell-extrinsic barrier mediated by the immune system are the main defenses of our body against oncogenesis. Only those cells, which escaped from both barriers, have the opportunity to develop into tumor. p53 tumor suppressor is widely recognized as a master regulator of cell-intrinsic anti-tumor defenses via induction of growth arrest, apoptosis, senescence, autophagy, and inhibition of cancer cells metabolism. Here, we show that, apart from well-characterized cell-intrinsic mechanisms, p53 also plays a role in modulating the cell-extrinsic anti-cancer defense.

In this study, we applied a set of p53-reactivating compounds, PRIMA-$1^{MET}$, nutlin, RITA, and a low dose of ActD, as research tools to address whether and how p53 can stimulate the immune response against cancer cells. We found that pharmacological reactivation of p53 enhanced the NK cell-mediated killing of samples derived from patients with metastatic tumors of a different origin, including melanoma, pancreatic, breast, colon, and lung carcinoma, as well as established lines, derived from different carcinomas, osteosarcoma and lymphoma.

We further demonstrated that this effect is due to the induction of ULBP2, a ligand of NK cell receptor NKG2D, an important component of the front-line immune defense against infectious diseases and malignancies.

Furthermore, we found that the molecular mechanism controlling NKG2D ligand transcription was p53-dependent, but ATM/ATR-independent. Using several approaches, we identified the binding of p53 to its RE within the first intron of *ULBP2* gene which is required for the activation of its expression, thus establishing *ULBP2* as a *bona fide* p53 target gene and suggesting a direct effect of p53 on stimulation of anti-cancer immune response.

However, stabilization of p53 by different agents is necessary, but not sufficient for binding of p53 to *ULPB2* gene and ULPB2 induction. In spite of a similar extent of p53 stabilization, different p53 activating compounds have distinct effect on ULBP2 expression.

The binding of p53 to its specific RE in DNA can be determined by the epigenetic modifications of the promoter. We found that the p53 RE in *ULBP2* gene is

highly methylated in cancer cells, which prevents p53 binding. As shown in Fig. 11, de-methylation of the p53 RE in *ULBP2*, achieved through repression of DNA methyltransferases (DNMTs), is required for the interaction of p53 with its binding site and the subsequent induction of ULBP2 by p53.

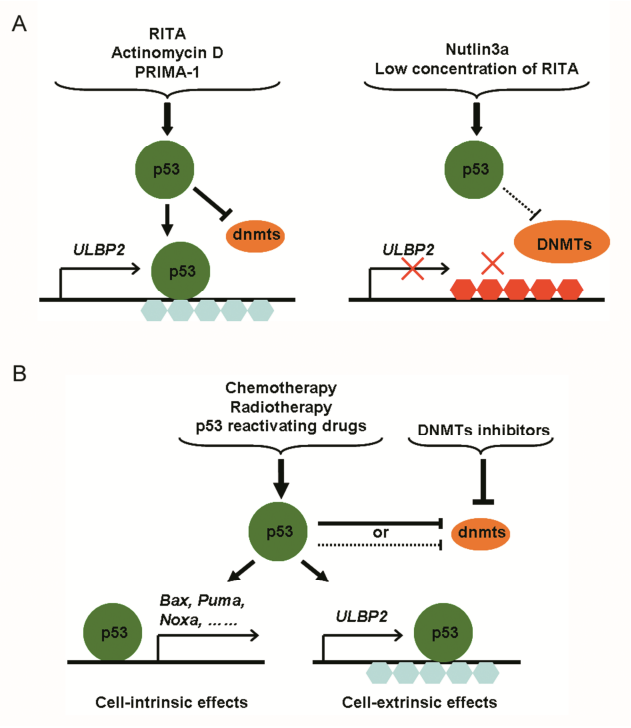


Figure 11. Model illustrating the mechanism underlying transcriptional control of *ULBP2* by p53 and proposed therapeutic implications. (A) p53 activated by PRIMA-1$^{MET}$, Actinomycin D, and RITA, but not Nutlin3a and low concentration of RITA, can induce de-methylation of its RE within the first intron of *ULBP2* gene via the repression of expression of *DNMT*'s. This allows the interaction of p53 with its RE and the subsequent induction of *ULBP2* transcription by p53. ⬣, non-methylated DNA; ⬣, methylated DNA. (B) Proposed therapeutic benefit of combined administration of p53-activating agents and DNMT's inhibitors.

Our studies provide a molecular evidence for the direct transcriptional control of immunosurveillance upon pharmacological restoration of the p53 function. This new facet of p53 reactivation - induction of genes invoking immune response along with the regulation of genes promoting apoptosis or growth arrest in cancer cells - may increase the probability to achieve a durable therapeutic success.

Co-operation with transcription factors, such as STAT3 and Sp1, have been shown to be important for p53-mediated transcription and biological outcomes (paper I and paper II). It is plausible that different composition and /or state of p53 cofactors which associate with p53 upon different stimuli confer the heterogeneity of p53 response. Since there are more than hundred known p53 partner proteins which can modulate p53-mediated regulation of gene expression, high throughput approaches are required to identify the bars of the code, which confer the differential regulation of immune response upon different p53-activating molecules.

# 3 CONCLUDING REMARKS

One of the issues our lab focusing on is to use small molecules to address the mechanisms of cell fate decisions by p53. The outcomes of the administration of these compounds differ depending on a type of compound or cell types and are hard to predict due to the complexity of p53 network. Thus, the elucidation of the mechanism by which p53 governs apoptosis is urgently needed. To address this issue, integrated study of several high throughput approaches was employed, since it allows us to study the complex network as a whole.

The transcriptional activity of p53 may be determined by the level of p53 in cells, the selection of binding site in chromatin, recruitment of cofactors and the epigenetic modification of chromatin (Beckerman and Prives, 2010). Our analysis of genome-wide p53 occupancy reveals that, regardless of stimuli, the level of p53 and biological outcomes, most of p53 binds to the same response elements sites in chromatin, which suggests that p53 binding to promoters *per se* is not sufficient to regulate transcription and emphasizes the importance of co-operating and antagonizing transcription factors of p53 in the determination of p53-mediated transcriptional program. To identify factors crucial for p53-mediated apoptosis, genome-wide shRNA screen was performed, which identified Sp1 as a central modulator of p53-mediated apoptosis. No effect of Sp1 on p53-mediated cell cycle arrest was detected, indicating that p53 regulates apoptosis and cell cycle arrest via distinct mechanisms. Besides, our combined analysis of chromatin occupancy data and gene expression data uncovers that Sp1-p53-mediated pro-apoptotic transcriptional repression is required for efficient induction of apoptosis by p53. Furthermore, our data suggests that Sp1 is also under the control of p53-MDM2 feedback loop, which tightly controls p53-mediated apoptosis in cells.

Further characterization of the mechanism by which Sp1 and p53 co-regulate p53-mediated transcriptional repression is needed, including the post-translational modification of Sp1 and p53, the recruitment of DNA-binding complexes and even the epigenetic modification of chromatin, since many chromatin modifying enzymes are regulated by Sp1 and p53.

Interestingly, our integrated high-throughput analysis also reveals that several genes and pathways involved in immune response can also be affected by p53 reactivation. Among them, ULBP2, a ligand of NK cell receptor NKG2D, can be induced by RITA activated-p53, but not by nutlin activated-p53, which supplies us a tool to address the underlying mechanism by which p53 regulates the expression of *ULBP2* and subsequent NK cell-mediated killing of human cancer cells. Using several approaches, we identified *ULBP2* as a direct transcriptional target of p53 and the induction of *ULBP2* expression requires the exposure of p53-binding region in *ULBP2* gene, which is achieved by p53-mediated repression of *DNMTs*.

This study provides a molecular evidence for the direct transcriptional control of immunosurveillance upon pharmacological restoration of p53 function. Our integrated high-throughput analysis shows that both innate and adaptive immune responses can be altered by p53 reactivation. However, the exact mechanisms of the regulation of immune responses by p53 remain unknown. Further investigation of the biological significance and the molecular mechanism of the regulation of immune

components by p53 may help to achieve a durable therapeutic success via the induction of anti-tumor immune response along with the promotion of apoptosis in cancer cells.

Our studies presented in this thesis contribute to the understanding of the mechanistic basis of p53-mediated intrinsic and extrinsic tumor suppression and paves a way to the design of new strategies for more efficient p53-based therapies.

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# 6 PUBLICATIONS AND MANUSCRIPT